International Journal of Botany
and Research (IJBR)
ISSN(P): 2277-4815; ISSN(E): 2319-4456
Vol. 4, Issue 3, Jun 2014, 31-38

BIOLOGICAL EFFECT OF THE ENTOMOPATHOGENIC FUNGUS METARHIZIUM ANISOPLIAE VARIETY ACRIDUM AGAINST THE HOUSE-MOSQUITO CULEX PIPIENS

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ABSTRACT

The effect of the entomopathogenic fungus *Metarhizium anisopliae variety acridum* on the *Culex pipiens* mosquito in the region of Régaïa (East Algiers) is reported for the first time in the laboratory conditions.

We treated the different instars larvae, pupae and eggs with different concentrations of the fungus solution C1=3.10^3 spores/ml, C2=3.10^4 spores/ml, C3=3.10^5 spores/ml and C4=3.10^6 spores/ml. The main values of mortalities were collected the first, the second and the third day after treatment.

The results showed that the *M. anisopliae var. acridum* is a promising biological pesticide against the *Culex pipiens* particularly on nymphs and eggs and it could be an alternative to chemical pesticides.

KEYWORDS: *Culex pipiens*, vecteur, Biologic Control, *Metarhizium anisopliae var acridum*

INTRODUCTION

Vector-borne diseases are complex epidemiological cycles related to the mode of indirect transmission; they are among the most important problems in human and animal health. These diseases occur when the pathogen is transmitted from one infected individual to another through a hematophagous arthropod called vector [1, 2]. Among these vectors, mosquito causes nuisance to human beings, birds and mammals [3]. They deposit saliva in the skin when they take blood that is necessary for egg production, and this permit itching [4].

Culex pipiens (*Diptera: Culicidae*) is a vector of important human diseases, such as West Nile virus, Rift Valley Fever virus, Malaria and Bancroftian flariasis which affect more than 700 million people annually [5]. This insect has a wide distribution throughout tropical and subtropical areas [6] and in Algeria *C. pipiens* is the most common species of mosquitoes in urban and rural areas [7].

The control of mosquitoes was routinely done using the mosquito's nets and repellents. Moreover, people have attacked mosquitoes with insecticides [8] and continued applications of organophosphates such as temephos and fenthion and insect growth regulators such as diflubenzuron and methoprene [6]. These methods have disrupted natural biological control systems and have resulted in the widespread development of resistance [3, 9]. These problems have warranted the need for developing alternative strategies using eco-friendly products for mosquito control [10].

The use of natural enemies of the mosquito appears to be an alternative approach to the systematic failure of insecticides [11]. Then, entomopathogenic microorganisms play an important role on these methods of biological control programs [10]. They have the advantage compared to most chemicals, to be generally specific to locusts without affecting other natural enemies [12].

However, the control efficacy of these entomopathogens is variable because of unfavourable and fluctuating environmental conditions and intrinsic factors [13]. Among entomopathogens, fungi have attracted a lot of attention as biologically based pesticides. *Beauveria bassiana* (Bals.-Criv.)Vuillemin and *Metarhizium anisopliae* (Metschn.) Sorokin are two of the most common species of entomopathogenic fungi investigated. Both *Beauveria* spp. and *Metarhizium* spp. are cosmopolitan anamorphic genera of soilborne facultative necrotrophics arthropod-pathogenic fungi [14].

In this study we evaluate the biological effect of the entomopathogenic fungus *Metarhizium anisopliae Var acridum* on the different developmental stages of the *C. pipiens.*

METHODS

Rearing Mosquitoes

Culex pipiens eggs, larvae and pupae were collected from sites of a cynegetic lake center in Reghaia of East Algiers (3° 19' and 3° 21' Est, 36° 45' and 36° 48'N) and reared in the Laboratoire des Systems Vectoriels de l'Institut Pasteur d'Algérie (Algeria). Larvae were reared in plastic trays with tap water. They were maintained at 27°C and about 75% of relative humidity, under 14:10 light and dark photoperiod cycle. The larvae were fed with biscuit. Pupae were transferred from the trays to a cup containing tap water and placed in screened cages (30x30x30 cm) where the adult emerged. After emergence, female mosquitoes obtained blood meal from an anesthetic guinea pig according to the protocols instructions, while male mosquitoes were fed on a 10% sucrose solution. Then egg-masses were kept in water to continue next instars larvae [7]. Pictures of the larvae pupae eggs and adults were taken in the laboratory with a camera (Samsung Digimax L85) fixed on a microscope (LEICA GLS X 150).

Culturing the Fungus and Preparation of Stock Solution and Dilutions

The fungal material was provided by l'Institut National de Protection des Végétaux d'Alger (INPV) in the form of an oily suspension containing spores of the *M. anisopliae var. acridum* fungus. 1ml of this suspension was centrifuged and seeded in Petri dishes containing Sabouraud chloramphenicol culture medium. The dishes were incubated over 5 days at a 28 °C temperature. The fungus appeared on the 5th day as whitish colonies and became greenish on the 7^{th} day. The purification was achieved by sub culturing in the same medium isolation. The fungal obtained isolates were identified by the study of macroscopic and microscopic aspects and were subsequently used for preparing solutions.

Several colonies of the fungus obtained from young cultures of 9-15 days were introduced in a flask containing 500 ml of sterile saline. Then, a barometer of the reagent and two drops of Tween 80 were introduced into the flask, so the solution was left on a vortex at a speed of 1500 rpm for 30 minutes to allow a good release of spores. The counting of spore was done under a microscope with Malassez cell [7].

Bioassays on Larvae, Pupae and Eggs of *C.pipiens*

To administer the solution containing *M.anisopliae* we chose the contact and ingestion mode recommended by various researchers, because it is a preferred route of infection and current for entomopathogenic fungi [15]. Bioassays were performed with the four larval stages, pupae and eggs of *C.pipiens* using concentration from 3.10^3, 3.10^4, 3.10^5 and 3.10^6 spores/ml of the *M.anisopliae.* A minimum of 25 larvae per concentration were used for all the experiments and these were repeated four times [7].

For the mortality studies, 25 larvae from each of the four instars and pupae were introduced into 250 ml plastic beaker containing various concentrations of the *M.anisopliae*. A control was maintained by putting larvae in clean water.

The treatments were repeated four times, and each replicate set contained one control [7]. The same solutions at different concentrations were used to assess the biological activity of *M.anisopliae* on larval duration. Eggs were put to hatch and the total larval duration (by days) was calculated from hatching to the pupation period referring to the control.

Statistical Analysis

In order to confirm the effectiveness of treatment, we made an analysis of variance using the software "Excel Stat." Data from biology, mortality, and effective concentration were subjected to analysis of variance ANOVA. Mortality percentages were determined and corrected using Abbott's formula (1925).

RESULTS

Dose-response relationship was determined for *M.anisopliae* applied for 24h, 48h, and 72h after treatment to the four instars larvae, pupae and eggs of *C.pipiens* and significant differences were observed.

Metarhizium anisopliae Effect on Mortality of Larvae and the Larval Duration

We began recording mortalities in the four larval stages L1, L2, L3 and L4 from 24h of treatment with the four concentrations 3.10^3, 3.10^4, 3.10^5, 3.10^6spores/ml, all tests showed increased mortality with increased concentration. Time of exposure reached after 72h of treatment with C4: 47% for L1, 50% for L2, 24% for L3 and 40% for L4.

The differences between controls and the group treated with different concentrations was significant $p=10^{-4} < 0$, 005. The cumulative percentage of mortality caused by *M.anisopliae* at various concentrations applied after 24h, 48h and 72h to larvae and pupae of *C.pipiens* was represented on (Figure 1). Low mortality rates of larvae in the controls were related to variations in the laboratory conditions like temperature, oxygen and nutrient.

Pupae

We have observed in pupae treated by *M.anisopliae* a mortality before emergence after 24h 48h and 72h of treatment with C1, C2, C3 and C4 (Figure 1). The average rate of deaths was augmented with the increased concentration used. It was achieved with C4: 63% after 24h, 78% after 48h and 82% after 72h of treatment. There was an extension of the pupal period when an occasional interruption of the emergence happened; this interruption was followed by the death of individuals and sometimes the death of adults after emergence. Some pupae specimens emerged with deformed wings and a proliferation of the fungus was noted on the bodies of the treated mosquito's (Figure 2). There was a significant difference between the control group and the treated pupae at different concentrations $p=10^{-4} < 0$, 005.

Eggs

C.pipiens' eggs were treated with C1, C2 and C3 presented after 24h and 48h of treatment formation of fungus; a very thin mycelium on the surface without hatching. The fungus developed after 48h and covered the entire tray with a partial outbreak after 72h. While eggs were treated with C4, after 72h they immersed in water and the fungus was multiplied in the surrounding area of the eggs (Figure 2).

The result of the estimation of the larval and the pupal duration showed an extension of the period until 23 days. To confirm the effectiveness of treatment we have followed the normal cycle of a tray of eggs (control), which revealed a larval duration of 19 days.

DISCUSSIONS

In this study, we proved once again that the use of biological methods to fight against pests can be achieved without using chemical products. The study of the effect of bio-pesticide on mortality of larvae, pupae and eggs of *C.pipiens* has showed that treatment with this product with the two modes of infection: ingestion and contact, has marked a significant efficacy compared to witnesses (p <0.05).

Various researchers have shown interest in studying the biological activity of the fungi, thereby; it is the subject of intensive research devoted to two main objectives. The characterization of virulence factors that can improve the infection process and the mechanisms of host specificity is one of the most important advantages of the biological control [16] and because this fungus can persist in soil or in insect cadavers for a long time, even under unfavorable environmental conditions [17].

M.anisopliae infects insects by contact, not ingestion, which is similar to the action of many chemical insecticides [15, 18] but it was confirmed that the infection of *M.anisopliae* in locust is by both ingestion and contact modes [19]. Because the infection occurs when the insect comes into contact with the conidia, it may also occur through the respiratory system [20].

We have observed that *M.anisopliae* was significantly more toxic after 72h of treatment with C4 and it was more aggressive on pupae and eggs compared to the larvae. Larvae, pupae and eggs were immersed in a conidial suspension to 10^6spores/ml. This concentration was selected as the highest, based on results of work on the sensitivity of a predator Culiciphage, *Toxochynchites amboineusis* to the entomopathogenic hyphomycete *M.anisopliae*, and to avoid high toxemia concentrations [15].

Our results show that 50% of L2 died after 72h of treatment with C4, we hypothesized that this mortality was due to mycotoxins produced by *M.anisopliae* which are lethal to mosquitoes in the larval stage [21]. To track the host cuticle, *M.anisopliae* used a synergistic strategy of hydrolytic and lipolytic enzyme secretions; for example (proteases, chitinases, lipases, andesterases) and mechanical pressure to breach the first and the most important host barrier to infection [22].

It was observed that the strain of *M.anisopliae* is the most aggressive strain of *C.pipiens* [23]. Integumentary and immune barriers do not allow mosquito larvae to resist infections, because this organism has a dual mode of action: toxemic effect at high doses > (10^7spores/ml) and parasite below this threshold [15], which may explain the low mortality. When the dose is less than $3x10^6$ spores/ml, the fungus follows a parasitic way to infect the haemocoel. Larvae can also eliminate the fungus with molting because the fungus can't attack the new integument [24].

Mortality happened before emergence in the early pupal stage after 24h of treatment with high doses of C4. Gradually, at the end of the pupal stage, the morphology of the adult contained in the exuvia is becoming more and more see transparent. The increase in internal pressure causes a tear of the medial dorsal cuticle of the carapace, allowing the emergence of the speciman [25] but after 72h with C4, this is followed by a blockage of the adult who is paralyzed and this causing its death. *M.anisopliae* produces destruxins causing paralysis and insect death, days after infection depending on species and size [20]. Microbial enzymes secreted by this fungus can act in the normal formation of the cuticle resulting in an abnormal and lethal molting of the Locust [26] which may be compatible in the case of mosquitoes confirmed by the results observed.

The effects of fungal infection on the hatching of eggs were also significant, the fungus develops in water and covers the entire tray and we observed a partial outbreak after 72h. *M.anisopliae* var. *acridum* reduced egg hatching by 40%, confirming our results, while eggs treated with the highest concentration after 72h, it immersed in water and this was due to the development of the fungus that blocked the floatation of the tray [27].

CONCLUSIONS

This research represents a significant contribution to understanding the effect of the bio-pesticide *M.anisopliae variety acridum* on the mosquito *C.pipiens*.

We have focused our study on the symptomatology and the mortality of different specimens of larvae, pupa and eggs, and the effect was manifested by rates of mortality that grew by increasing the concentration used. For performing processing on a large scale, further studies on extensive experiments under controlled conditions are needed to control all parameters such as dose, method of inoculation, the size, the sex of larvae and a great knowledge of the fauna of the site.

ACKNOWLEDGEMENTS

We are very grateful to all personnel of Service d'Ecologie et des Systèmes Vectoriels, Institut Pasteur d'Alger, (Algérie), especially Assia Beneldjouzi, Nacera and korba Anissa for their assistance.

Authors' Contributions

BI and HF conceived the study. BI, SI, and RA participated in collecting samples, CN, SI, conducted data analysis. SI, RA, HF and BI interpreted the results, and reviewed the initial and final drafts of the manuscript. All authors read and approved the final manuscript.

Competing Interest

The authors declare that they have no competing interests.

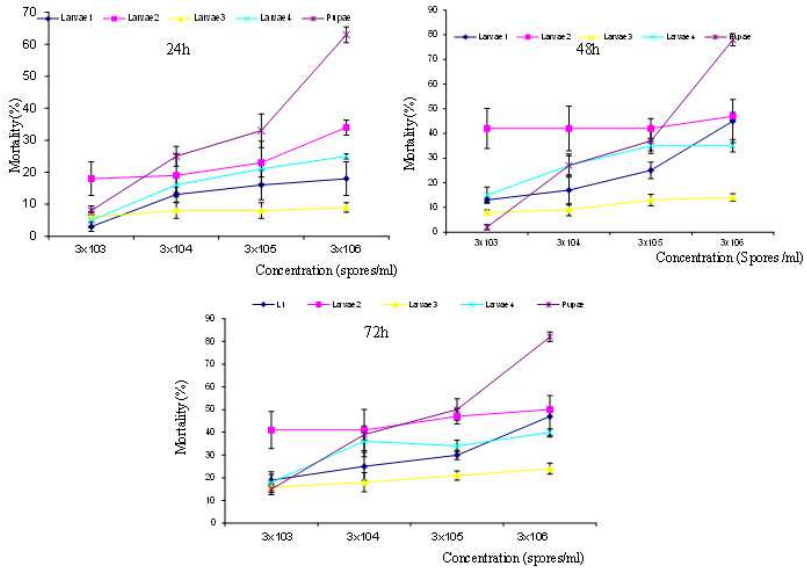

Figure 1: The Cumulative Percentage of Mortality Caused by *M. Anisopliae a*t Various Concentrations Applied for 24h, 48h and 72h to Larvae and Pupae of *C. Pipiens*

Figure 2: Symptomatology of Larvae, Pupae and Eggs of *C.pipiens* after Treatment with *M.anisopliae var. Acridum.* GX40

a) - Deformed Nymph died after treatment compared to the control a'.

b) - Paralysis of adult treated with interruption of the emergence.

c) - Death of adults after emergence.

d) - Adults with atrophied wings compared to the control d'.

e) - Mycelium covering two adults died after treatment with *M.anisopliae*.

f) - The egg-tray of *C.pipiens* treated after 48 hours.

g) - Partial- eclosion of the tray treated after 72 hours.

h) - Treated egg tray with C4 after 72 h shows immersion in the water without hatching.

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